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Phenotyping the haemostatic system by thrombography—potential for the estimation of thrombotic risk

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ABSTRACT

The aim of this paper is to review the thrombogram and its use for phenotyping the haemostatic system. The thrombogram can be readily obtained through Calibrated Automated Thrombography (CAT), using a commercially available fluorometer, dedicated software (ThrombinoScope®) and a calibrator. Here we explore the possibility to use platelet-rich plasma (PRP) triggered with a low amount of recombinant human tissue factor (~ 0.5 pM) and also explore the function of the protein C system by adding activated protein C (APC) or soluble recombinant thrombomodulin (TM). Examples are shown: inherited antithrombin (AT) and protein C deficiencies, and antiphospholipid antibodies.

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Introduction

Evidence has accumulated over years that traditional clotting assays, while quite informative for marked haemostatic defects, fail to indicate in-

creased coagulability of patients suffering from thrombosis. A striking example of this failure is seen in patients with lupus anticoagulants (LA) where the anticoagulant activity observed by in vitro tests is in sharp contrast with the increased associated thrombotic risk [1–4].

The generation of thrombin is the final common path of the blood coagulation process and the sequence of events leading to activation and inhibition processes has been referred to as the initiation,

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propagation and termination phases [5]. To ensure reproducible results with standard clotting assays, platelet-poor plasma (PPP) and either extensive contact activation or a large excess of tissue factor are traditionally used. Such strong triggers must be deemed physiologically irrelevant. Moreover, clotting, the end-point of these assays, coincides with the onset of the thrombin burst (propagation phase), a moment at which >95% of thrombin is still to be generated [5,6]. Indeed, during recent years the need for a general function test that reflects the complexity of thrombin formation and inhibition has been emphasized [7,8]. Several such tests have been proposed, some using clot formation and changes in the mechanical properties of the clot, such as the thromboelastography [9], others based on measuring thrombin generation.

Thrombin generation has been known for a long time to be a valid physiological function test [10,11]. The technique was revived in the 1980s [6] and is now being increasingly utilized in a number of different forms [12–16]. Its widespread application has been hampered by the fact that in its classical form it requires about one man-hour of skilled laboratory work to obtain a curve. The Calibrated Automated Thrombogram (CAT), using a fluorogenic thrombin substrate and continuous comparison to a simultaneously run calibrator [17] relieves this limitation. CAT also allows measurement in platelet-rich plasma (PRP) [18].

The CAT methodology is sensitive to hypercoagulability and bleeding disorders [19,20]. We have previously reported that the CAT methodology can be conveniently implemented in clinical research and that, in some instances, frozen-thawed PRP, which allows the design of large clinical studies with a core laboratory, can be used to assess the effect of the protein C system and known risk factors for thrombosis [21]. In LAPatients, we demonstrated activated protein C (APC) resistance despite the occurrence of long initiation phases [22]. This paper will present typical examples of phenotypic assessment of the haemostatic system in inherited or acquired prothrombotic disorders with focus on antiphospholipid antibodies.

Materials and methods

Reagents

Bovine serum albumin (BSA) and all chemicals were from Sigma (St. Louis, MO, USA). Recombinant human tissue factor was either a kind gift from

Dade Behring (Marburg, Germany) or the commercially available reagent Innovin from Dade Behring. The fluorogenic substrate of thrombin, Z-Gly-Gly-Arg-AMC, was from Bachem (Bubendorf, Switzerland) and the calibrator with a constant, known, thrombin-like activity from Synapse (Maastricht, The Netherlands). Soluble recombinant human thrombomodulin (TM) was kindly provided by Asahi (Japan) and APC was prepared in-house as previously described [23].

Blood and plasma

At venipuncture, blood was either drawn by vacuum into Vacutainer® tubes (Becton Dickinson) or by gentle suction into Monovette® (Sarstedt) syringes, 1/10 volume of 0.129 or 0.106 M sodium citrate, respectively. It was centrifuged at $194 \times g$ for 10 min at room temperature to obtain PRP and twice at $2500 \times g$ for 10 min at room temperature to obtain PPP. Platelets were counted using a Micros 60 ABX model (Montpellier, France) and adjusted to 150×10^9 platelets/L with autologous PPP. PRP was used within 90 min after venipuncture unless otherwise stated.

Calibrated automated thrombin activity measurement

Thrombography was performed at 37 °C according to Hemker et al. [12,19] in a microtiter plate fluorometer (Fluoroskan Ascent, ThermoLabsystems, Helsinki, Finland) using the dedicated software program (Thrombinoscope®, Synapse), as previously reported [21].

Briefly, coagulation was triggered by recalcification in the presence of 0.5 pM recombinant human tissue factor. Experiments were carried out in the absence or in the presence of APC or TM at various concentrations. The molar amount of thrombin present in clotting plasma was calculated from the signal of wells in which thrombin was generated and the calibration signal [19]. The total amount of thrombin activity (known as the endogenous thrombin potential—ETP) was assessed as the area under the curve.

Results

Most results are basically given as phenotypic patterns: superimposed tracings of thrombin activity as a function of time with PRP. Five concentrations of APC ranging from 0 to 65 nM (corresponding to the activation of 100% of plas-

ma protein C) were studied. However, with the exception of patients with antiphospholipid antibodies (aPL), and for clarity's sake, only results obtained with 25 nM APC are presented. This concentration was chosen assuming a similar circulating level of APC in human plasma as those reached in baboons infused with thrombin [24] and it induces ~70% inhibition of ETP with normal PRP [21].

Effect of preanalytical conditions and storage

To evaluate the influence of preanalytical conditions on thrombin activity in the absence or in presence of the protein C system, we compared the effect of vacuum during the drawing of blood and of the time before centrifugation. It is seen in Fig. 1 that using vacuum can lead to platelet activation and decrease in APC sensitivity. These changes are seen shortly after blood collection and centrifugation and show significant variability between donors (Fig. 2).

Examples of typical inherited or acquired hypercoagulability disorders

A typical phenotype of a control healthy subject is shown on the left for each of the following examples.

Fig. 3 shows a typical profile of inherited antithrombin (AT) deficiency (plasma level: 62 U/dL). As expected, the most conspicuous differences lie in the peak of thrombin with a higher value than normal and the termination phase with a slower decay of thrombin activity than with the control. As illustrated in the insets, the thrombin

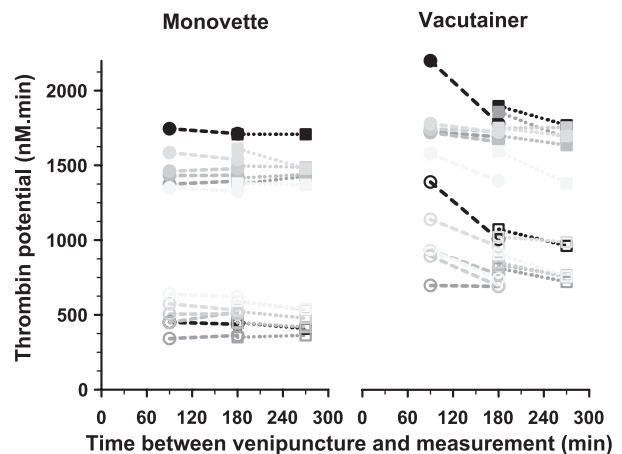


Figure 2 Effect of time before centrifugation of blood and PRP storage on thrombin activity. PRP preparation was initiated either 30 min (circles) or 120 min (squares) after blood withdrawal; two recordings of thrombin activity were started at 60 and 150 min after the onset of PRP preparation, and are linked with short dashed and dotted lines, respectively. Data obtained for six healthy individuals in the absence or presence of 25 nM APC are presented.

potential is high when AT is deficient. APC response (a 43% decrease in ETP with 25 nM APC) falls within our control range obtained with 20 healthy individuals (median inhibition of ETP by APC: 66%; range 43–77%).

A typical profile of inherited protein C deficiency (plasma level: 50 U/dL) is shown in Fig. 4: normal pattern of the effect of exogenous APC but hardly any effect of added TM. The concentration in TM was chosen in accordance with earlier preliminary data showing a similar ~70% decrease in ETP with 25 nM APC or with 10 nM TM [21]. Although the ETP is within the normal range

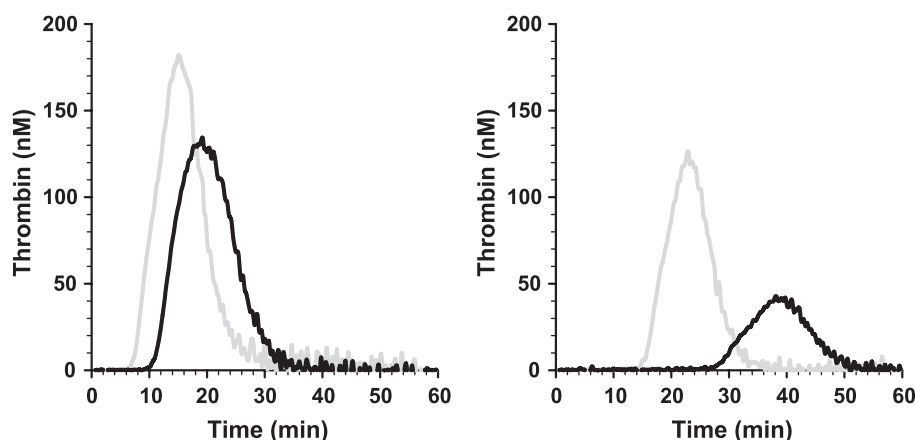


Figure 1 Effect of blood collection on thrombin activity. Thrombograms observed with PRP prepared 30 min after blood collection into Vacutainer (gray lines) or Monovette (black lines) tubes in the absence (left) or presence (right) of 25 nM APC.

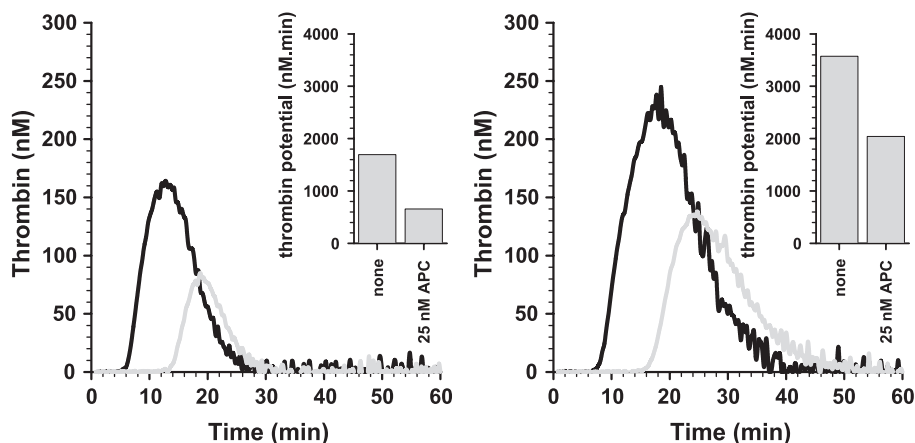


Figure 3 Typical phenotype of inherited antithrombin deficiency. Left: control; right: patient with AT deficiency (AT plasma level: 62 U/dL). Results are means of triplicate in the absence (black) or presence (gray) of 25 nM APC.

in the absence of APC, the defect in protein C leads to elevated values when the endogenous APC system is triggered.

Typical phenotypes of patients with aPL are shown in Fig. 5. We previously reported prolonged initiation phases of thrombin generation in LA-patients together with a marked inability for APC to diminish thrombin activity [22]. In fact, thrombograms of patients B, C and D (Fig. 5) are representative of the three types of profile that we observed with 30 aPL patients (data to be published). Type 1: similar to the control (B); type 2: decreased APC sensitivity (C) and type 3: almost no inhibitory effect of added APC on ETP while it did prolong the lag-phase (D). These effects are best rendered by the ETP of the uninhibited sample (1696, 1628, 1334 and 1253 nM.min for the control, patients B, C and D, respectively) and the concentration of APC that would give 50% inhibition—IC₅₀—

APC (15.3, 12.5, 36.8 and >65 nM for the control, patients B, C and D, respectively). Even though the IC₅₀ concentration in APC is not reached with the APC concentrations used for patient D, we did not want to increase APC concentrations beyond physiological levels.

Discussion

Calibrated automated thrombography has been proposed to identify hypercoagulability [17]. We confirm the potential utility of such an integrative assay for evaluating the haemostatic phenotype of an individual, especially because the function of the protein C system can be easily probed by adding either exogenous APC or TM that, when thrombin is formed, activates endogenous protein C.

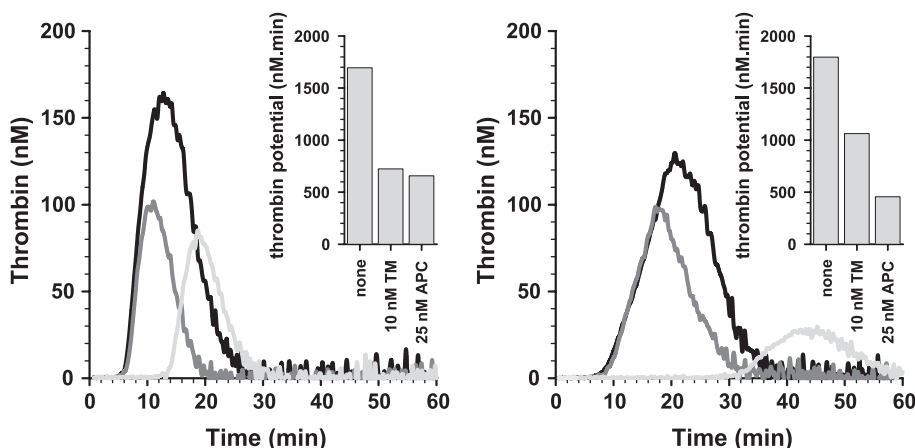


Figure 4 Typical phenotype of inherited protein C deficiency. Left: control; right: patient with protein C deficiency (protein C plasma level: 50 U/dL). Results are means of triplicate in the absence (black) or presence of 10 nM TM (dark gray) or 25 nM APC (light gray).

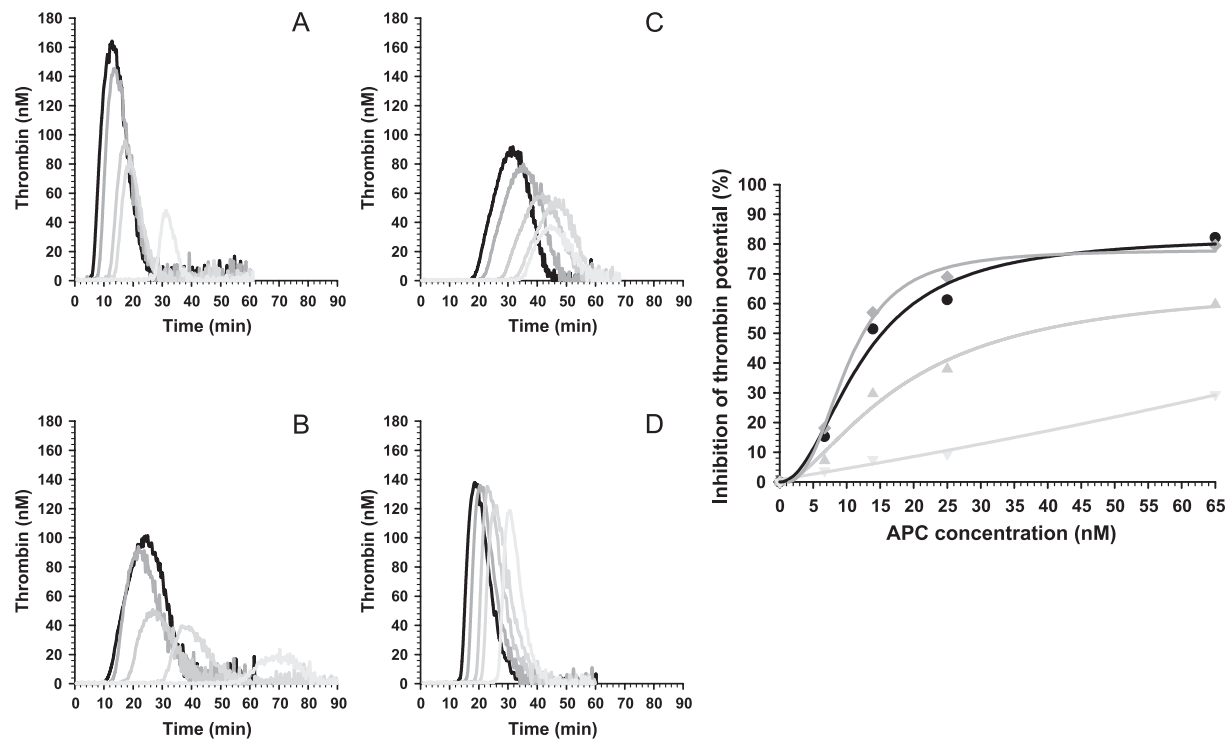


Figure 5 Typical phenotypes of aPL patients. Panel A: control; panels B to D: three typical aPL patients. Added APC concentrations: 0, 6.7, 13.9, 25 and 65 nM. Right panel: the inhibition of thrombin potential by APC was illustrated for the matched control (black) and for patients B (dark gray), C (gray) and D (light gray). ETP values (nM.min): (A) 1696; (B) 1628; (C) 1334; (D) 1253. IC₅₀-APC values (nM): (A) 15.3; (B) 12.5; (C) 36.8; (D) >65.

Ideally carefully prepared fresh PRP should be used to avoid artifacts due to accidental platelet activation or to platelet aging. Our findings confirm that the conditions of venesection are important in particular with respect to PRP stability on the one hand and APC sensitivity on the other hand. It is interesting to note that storage of total blood collected with minimal suction and subsequently prepared PRP at room temperature for more than 180 min had no effect on the thrombogram (Fig. 2). We nevertheless do not recommend this procedure because (i) there are large individual variations in behaviour, possibly also dependent upon the use of medication and (ii) we have seen diminution of the sensitivity of the platelet to antagonists of platelet receptors.

Although for LA-patients the initiation phase is prolonged by "definition" (otherwise there would be no anticoagulant action), the maximal rate of thrombin generation and the thrombin potential value are often within the normal range.

Our data indicate that well-established conditions associated with a persistent thrombotic risk show a gain of function phenotype, i.e. increased thrombin potential in the absence and/or in the presence of added APC or TM. The thrombin potential in the absence of APC is not necessarily signif-

icantly abnormal for patients with inherited protein C deficiency or for patients with APL. Thus, the amount of thrombin generated with an activated protein C system could be more predictive of thrombotic risk than when only tissue factor is added.

A parameter directly related to the endogenous thrombin potential is the level of the α_2 -macroglobulin-thrombin complex in (defibrinated) "plasma" after thrombin generation is over. An APC sensitivity ratio (APCsr) can be calculated from such levels obtained in the presence and in the absence of APC. An association between APCsr and the use of oral contraceptives as well as with factor V G1691A polymorphism has been established but conclusions are controversial regarding the association of APCsr with venous thromboembolism [25,26].

APCsr vary with the concentration in APC and the choice of a concentration remains arbitrary. A peak circulating APC level of 23 nM was reported in baboons (concentrations in protein C and prothrombin in the normal human range) infused with thrombin [24] but the variability in the extent of protein C-to-APC conversion in vivo is unknown and a "physiological" concentration cannot be established.

We therefore opted to characterize the individual patients by two parameters, the uninhibited ETP and the concentration in APC that gives half maximum inhibition of the thrombin potential (IC_{50} -APC). We can speculate that there exists a value of the ETP, in the presence of a given amount of APC, that best represents the *in vivo* situation and therefore in itself is the indicator of the thrombotic risk. As we do not know what that amount might be we prefer to characterize the situation by the uninhibited ETP on the one hand and the IC_{50} -APC on the other as a measure of the APC-sensitivity.

Thrombin generation has been reported to be associated with a number of features of each coagulation factor [20,27]. A blood composition-defined propensity for any given individual to respond with a characteristic amount of thrombin work for a given appropriate tissue factor stimulus has been evidenced [16,19,21]. Thus, the thrombin potential in the presence of APC appears to integrate most variables with, however, the above-mentioned limitation on the choice of APC concentration. *Mutatis mutandis*, the same holds for the addition of soluble TM. Only carefully designed and conducted clinical studies can tell us whether our hypotheses are correct [28]. Thus a study program has been initiated. First the study of a sample of the general population will provide reference values. Second, a case-control study will estimate the risk of occurrence of first thrombotic event associated with hypercoagulability evidenced by thrombography in patients with an antiphospholipid syndrome or a systemic lupus erythematosus. Third, since a case-control design cannot sufficiently consider time-dependent effects both from the exposure variable and the potential confounders it will be followed by a prospective cohort study.

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